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(54) Proteolytic enzyme treatment of wounds

(57) A powder, which serves for covering and treatment of ulcerous and necrotic wounds consists of porous spherical cellulose with particle size 0.05 to 0.5 mm, which contains an immobilized protease e.g. chymotrypsine, trypsin or subtilisine.

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SPECIFICATION

Proteolytic Cover of Wounds

The invention pertains to a proteolytic cover of wounds in the form of a powder which serves for coating and treatment of ulcerous and necrotic wounds.

The powders consisting of various bases (starch, talc, etc.) and bactericides or bacteriostatics, in particular antibiotics, sulfonamides and antimycotics, are recommended for this purpose in the common pharmaceutical practice.

Recently, absorbing covers in the form of powders, which contain a hydrophilic polymer as a main component, proved suitable in the treatment of ulcerous wounds. Their effect consist in the removal of liquids from the surface of wound by sorption in a porous structure of the effective polymeric component and by suction of capillary forces into voids between particles of the powder layer. The exudate together with bacteria, substances causing inflammation, or also toxins are removed from the wound in this way. In particular, preparations based on crosslinked dextrans (B. S. Jacobsson et al., Scand. J. Plast. Reconstr. Surg. *10*, 65—72 (1976)) are used in medical practice. Promising results were attained also with polymer compositions based on cellulose, which contain a highly hydrophilic polymeric component, for example carboxymethylcellulose as an additive (H. Dautzenberg et al., Absorbing cover of wounds; Czechoslovak Patent Application PV 4129-82).

Further development of powders with the cleaning effect in treatment of ulcerous wounds turned attention to cellulose powders containing immobilized enyzmes of protease type (Collection of papers: Immobilized proteolytic ferments in heating of ulcerous-necrotic processes (in Russian), AN SSSR, Sibirskoe Otdel., Institut tsitologii i Genetiki, Novosibirsk, 1981). In this case, the enzymatic action is used to attain cleaning effects. The proteolytic covers of wounds cause a hydrolytic decomposition and dissolution of necrotic tissues and pus on the wound surface thus removing also the medium for growth of bacteria and interrupting suction of toxic products from the wound.

Practical utilization of the proteolytic principle requires a solution of numerous problems in the preparation of the efficient cover which would not develop the harmful side effects. This means, in human medicine, above all to prevent cellulose or derivatized cellulose from entering into blood circulation, because the human organism has not enzymatic systems for removal of such compounds at disposal. However, the preparations of proteolytic cover used so far employ only conventional cellulose types, i.e. ground ion-exchanging dusts with a fibrous structure containing fine fractions of dust which may penetrate into blood.

To attain the maximum efficiency of covers, the proteolytic principle should not be employed without using the previously known absorption principle at the same time. Recent solutions comprise, however, only the conventional type of cellulose with a high crystallinity and low porosity as the starting material, which provides covers with a relatively low absorption capacity.

In addition, such procedures for the immobilization of proteases in cellulose have to be found for the utilization of proteolytic principle, which would exclude a release of the enzyme from matrix and penetration of its soluble form into blood circulation, where it would develop an allergic reaction as an antigen.

The above said shortcomings in the utilization of proteolytic principle are removed in a new cover for wounds according to the invention.

An object of the invention is a proteolytic cover for wounds, which consists of spherical particles of diameter 0.05—0.5 mm, preferably 0.1—0.3 mm, based on a derivatized cellulose with immobilized enzymes of protease type selected from the group which comprises chymotrypsine, trypsine, and subtilisine. Another object of the invention is a method for producing the proteolytic cover for wounds from bead cellulose, prepared by the procedure according to Czechoslovak Patent No. 172,640, where the bead cellulose swollen in water, which never has been dried, of particle size 0.07—0.7 mm, preferably 0.14—0.4 mm, is perfectly freed of toxic contaminants by washing or distillation with steam, activated for bonding of the enzyme, modified by immobilization of proteases selected from the group comprising chymotrypsine, trypsine, and subtilisine, alternately washed with a buffer of pH 8.5 to 9.5 and a buffer of pH 4 to 5 until the washings have proteolytic activity zero, then washed with a buffer of pH 7.5 to 8.5, and dried in this medium to 0.1 to 15% of the residual water content in the dry substance, preferably by lyophilisation.

The regular spherical shape of individual particles, which form the new cover for wounds, and the chosen particle size and the distribution of particle size (0.05—0.5 mm, preferably 0.1—0.3 mm), warrant an easy handling both in production and in application, a smooth flow of powder during its spreading on wounds, and, in particular, meet the requirement to prevent penetration of cellulose into blood circulation.

The production of new cover advantageously employs the regenerated spherical cellulose prepared according to Czechoslovak Patent No. 172,640. Its advantage is a high porosity which facilitates the activation for enzyme binding and the immobilization of proteases. The porous hydrophilic character of the bead cellulose carrier is retained even after activation, immobilization and drying. Lyophilisation proved a suitable drying method which removes water in a considerate way with respect to the bonded enzyme and provides the dry derivatized cellulose with a sufficient absorption power.

The bead cellulose prepared by the procedure described in Czechoslovak Patent No. 172,640 is thoroughly freed of soluble portions, in particular of all impurities with toxic effects which cause its contamination during preparation (decomposition products of xanthogenate groups, residues of a disperse medium, e.g. of chlorobenzene). For this

purpose, it is washed with water at 50—90°C and/or with ethanol. An effective removal of chlorobenzene occurs by distillation with steam.

Several methods known from literature can be used to activate the cellulose for protease binding (Handbook of Enzyme Biotechnology, Ed. A. Wiseman; E. Horwood Ltd., Chichester 1975), but such methods should be chosen which form a sufficiently stable bond between enzyme and cellulose and do not contaminate the product with toxic compounds becoming effective during application of the cover, for example, by releasing or action in the direct contact. A suitable method is the periodate oxidation, which is relatively simple and easy to carry out and provides products of a sufficient porosity which exhibit a high absorption effect.

Various proteases are suitable for immobilization, particularly trypsine, chymotrypsine and subtilisine, but also thermolysine, papaine, and others. Binding of the enzyme to cellulose activated by periodate oxidation, i.e. the cellulose containing reactive dialdehyde groups, is carried out in two steps: the compounds of cellulose with the enzyme of a Schiff-base type are formed first, which are then stabilized by reduction of the unreacted aldehyde groups with sodium borohydride.

An important stage in the preparation of the new type of cover is the perfect removal of soluble portions containing proteases from the modified cellulose beads. It is attained by the repeated alternation of a weakly alkaline (pH 8.5—9.5) and a weakly acidic buffer (pH 4—5) in a static or column arrangement. For this purpose, they are suitable, e.g., the 0.1 M borate buffer containing 1 M NaCl (pH 9) and the 0.1 M acetate buffer containing 1 M NaCl (pH 4.5), or both these buffers without NaCl, which are applied on the cellulose product after immobilization until the liquid phase has a proteolytic activity zero. The alternation of buffers of lower and higher pH and variable ionic strength facilitates the elution of covalently nonbonded protein by the alternating suppression of electrostatic and hydrophobic interactions. To obtain a perfectly stable preparation with the immobilized enzyme, the beads are eventually washed with a borate buffer, e.g., of pH 8, and lyophilized in a suspension with this buffer. Under such conditions, the resulting product contains only the covalently bonded enzyme and no soluble portion of it, which could penetrate into blood during treatment and could become an antigen.

The proteolytic cover with immobilized chymotrypsine was tested in clinical practice. It exhibited the proteolytic activity to necrotic tissue, pus and fibrin and, at the same time, did not harm a healthy tissue at all. The cover was noted for the high absorption capacity in agreement with the hydrophilicity and porous structure of cellulose matrix. It sucked up the exudate from a wound, which contained cleaved tissue necroses and bacteria. Binding of the enzyme to carrier prevents its autolysis and loss of its activity during application. The extended proteolytic activity of chymotrypsine led to the release of necroses from wound in a large extent and to the stepwise cleaning of wound. The early infected secretion was actively absorbed between the particles of powder thus preventing the circumference of wound from maceration and reducing the nutrient surface for the growth of bacterial infection. The original combination of the proteolytic and absorption principles enabled to achieve the fast cleaning of infected necrotic defects, formation of clean granulations and fast healing of the wound.

It has been proved that the new cover is suitable for all kinds of ulcerous and necrotic wounds, including the surgical wounds healing per secundam, for abcesses, prolonged and nonhealing ulcers of various origin (varicose, X-ray and trophic ulcers), defects after decubital necroses, infected and necrotic defects after acral amputations, incisions and necrectomies at diabetic gangrenes and gangrenes of arteriosclerotic origin, carbuncles, ulcerous burns of the 2nd and 3rd degree, infected open fractures, treatment of amputation stumps, decomposed and ulcerous tumours. No harmful side effects were observed in the application of the new cover.

The following examples of performance illustrate the method for production of covers according to the invention and their applications without limiting the scope of invention.

EXAMPLE 1

Preparation of the Cover with Bonded Chymotrypsine

a) Introduction of Aldehyde Groups by Oxidation with Sodium Periodate

The bead cellulose swollen in water and never dried before with porosity P=90% (i.e. the overall content of pores in volume %), which was prepared according to Czechoslovak Patent No. 172,640, was used as the starting material. Cellulose was perfectly freed of impurities by washing with hot water (90°, 5 h, twentyfold excess) and by distillation with steam (4 h). The filtered cellulose (1000 g) was dispersed in 5 l 0.1 M sodium periodate and stirred at laboratory temperature for 45 min. After completion of the oxidation, the oxidized cellulose was immediately washed with about 20 l distilled water (on Buechner funnel), transferred into a column and washed with distilled water until the electric conductivity of effluent became equal to the conductivity of distilled water (overnight).

b) Binding of Chymotrypsine to Oxidized Cellulose

The washed oxidized cellulose (1000 g) was dispersed in 1 litre 0.1 M borate buffer (pH 9) containing 5 g chymotrypsine (the proteolytic activity of the solution was 5.35 $j_{A_{280}}$/min.ml as determined by means of a solution of denaturated haemoglobin, pH 8). The suspension was stirred at laboratory temperature. The progress of binding was followed as the decreasing proteolytic activity of the binding solution. After an hour, the proteolytic activity decreased to 0.1 $j_{A_{280}}$/min.ml and bonding was stopped by suction off the binding solution. The rate of chymotrypsine binding increases with increasing pH, however, the

solubilization of cellulose increases at the same time depending on time of oxidation. The chosen procedure was worked out on the base of numerous comparative experiments and compromises between the amount of bonded enzyme and a loss of cellulose caused by solubilization.

c) Reduction of Cellulose with Bonded Chymotrypsine to Stabilize Schiff Bond between the Carrier and Enzyme and Remove Unreacted Aldehyde Groups

The cellulose with bonded chymotrypsine was suspended in 1 litre 0.1 M borate buffer (pH 9) with dissolved 500 mg $NaBH_4$. The reduction was stopped after 20 min. of stirring at laboratory temperature by suction off the reducing solution and once more repeated in the same way with the new solution of sodium borohydride. The reduction was carried out twice by addition of the $NaBH_4$ solution of lower concentration to prevent the loss of proteolytic activity of chymotrypsine by the contingent reduction of disulfide bridges.

d) Washing and Lyophilisation of Cellulose with Bonded Chymotrypsine

The cellulose with bonded chymotrypsine was washed, after completion of reduction, alternately with 2 l 0.1 M borate buffer containing 1 M NaCl (pH 9), 2 l 0.1 M acetate buffer containing 1 M NaCl (pH 4.5), and further with the same volumes of both buffers without NaCl. The cellulose with bonded chymotrypsine was then transferred into a column, where it was washed with all above mentioned buffers repeatedly always until the proteolytic activity of effluent was zero. The cellulose with bonded chymotrypsine was eventually washed with 0.25 M borate buffer of pH 8 and lyophilised also in the suspension with this buffer. The obtained preparation contained 5 mg active chymotrypsine on 1 g dry cellulose. The comparative amino acid analysis of the prepared sample and a sample additionally washed with 6 M guanidine hydrochloride and distilled water proved that all chymotrypsine is covalently bonded to cellulose and therefore cannot penetrate into blood during application and become an antigen.

EXAMPLE 2

Preparation of the Cover with Covalently Bonded Trypsine

Immobilization of trypsine was carried out in the same way as in example 1. The obtained preparation contained 8.2 mg active trypsine in 1 g lyophilised cellulose. Because of its narrower specificity, trypsine cleaves proteins at lower number of sites (it cleaves proteins only behind basic amino acids lysine and arginine).

EXAMPLE 3

Preparation of the Cover with Covalently Bonded Subtilisine

The immobilization of bacterial proteinase was carried out in the same way as in example 1. The obtained preparation contained 11.3 mg active enzyme in 1 g lyophilised preparation.

EXAMPLE 4

The proteolytic cover with chymotrypsine bonded according to example 1 was applied to 7 patients for the period of 1 to 4 weeks with a very good effect at 6 patients. The effect is characterized by retreat of purulent secretion, cleaning of the defect base and release of adhering necroses of which those of larger extent were surgically removed by necrectomy. The defect was rinsed with hydrogen peroxide solution and 2 per mille solution of Chloramine before further application of the preparation. Vivid red granulations and epithelisation of the defects from circumference, followed by their reduction in size, were achieved by this procedure. A local application of Panthenol spray into epithelising defect was mostly used after the treatment was finished.

One case showed a decrease of purulent secretion, one fifth of the defect was cleaned to vivid granulations, but deeply reacting adhering necroses occurred in the remaining parts of defect in the terrain of chronic ischaemia caused by the combination of diabetic microangiopathy and oblitering arteriosclerosis of peripheral arteries, so that an insufficient blood supply of neighbouring tissues was the limiting factor of healing of the defect. Four weeks after the application of the preparation was stopped, the defect made progress in the sense of abcessing diabetic phlegmon.

Examples of Application of the Preparation:

1) J. T., man, age 72, report no. 8357/83; a defect of diameter 3 cm after amputation of the 4th toe of right leg for the diabetic gangrene with wet necroses on the bottom and a purulent secretion. After 2-week application of the preparation, predominantly clean granulations occur with isolated adhering necroses in the medial edge of wound, without purulent secretion.

2. V. S., man, age 60, report no. 6417/83; the state after amputation of the right shin for diabetic gangrene, where the defect of diameter 5 cm and depth 2.5 cm arose after abcessing phlegmon, which had necroses on the lower lateral part of amputation stump and also medially scabby granulations in the whole range of wound 10 cm long and 1 cm wide with purulen secretion. After 4-week application of the preparation, almost complete healing was achieved, except a defect of diameter 2 cm and depth 1 cm in the lateral part of stump with clean granulations and progressive epithelisation of the circumference.

3) F. N., man, age 66, report no. 3376/83; a defect after amputation of 1st to 3rd toes including the heads of corresponding metatarses for diabetic gangrene, size 5—4 cm, partly with scabby granulations and partly with adhering necroses; the depth of defect was in its lateral part up to 1 cm. After 4-week application of the preparation, clean granulations were obtained in the whole region of defect with the progressive epithelisation of circumference and reduction to diameter of 3 cm. The same patient, F. N., man, age 66, after amputation of the distal half of 4th toe including the head of 4th metatarse; a defect arose of diameter 3

cm with a scabby lower part; after a week application of the preparation, the defect cleanly granulates without secretion and with sound surroundings.

4) F. V., man, age 77, report no. 254/84, with the defect after amputation of the 2nd, 3rd and 4th toe for diabetic gangrene of size 5×4 cm and depth up to 3 cm with an abundant purulent secretion and extensive adhering necroses in the whole range of wound; after 4-week application of the preparation, clean granulations were formed in one fifth of the defect; the necrotic process remained without limits in other parts due to poor blood supply in leg periphery at the diabetic microangiopathy and oblitering arteriosclerosis, but the purulent secretion in the defect ceased; the progress in defect occurred first after the application of preparation was stopped.

5) PhMr. M. K., man, age 56, report no. 9568/83, with the defect after incision of an abcessing phlegmon of the interphalangal joint of the 1st toe, which reached intraarticularly, of diameter 2.5 cm, with necroses and purulent secretion; after a week application of the preparation, the secretion receded, the defect was cleaned to clean granulations and the surrounding of defect get calm.

6) M. P., woman, age 78, report no. 1994/83, with a defect after amputation of the 1st and 2nd toes including the heads of corresponding metatarses for diabetic gangrene of size 5×3 cm, with a scabby lower part, adhering necroses and purulent secretion in the distal field of wound. After 4-week application of the preparation, clean granulations were obtained in the whole range of wound, the purulent secretion was suppressed and the defect was reduced to 3×2 cm by the advanced epithelisation.

7) M. T., woman, age 77, report no. 17741/83, with two defects in an amputation stump of shin after amputation for a diabetic gangrene followed by the abcessing phlegmon of stump, which caused liquefaction of the wound in whole region and defects of the size 10×2—4 cm with a purulen secretion, scabby granulations and extensive necroses. After 4-week application of the preparation, the granulations became clean, necroses were released, the secretion suppressed and epithelisation advanced from the circumference of the defects so that these were medially reduced to diameter of 3 cm and laterally to diameter of 1.5 cm.

CLAIMS

1. Proteolytic cover for wounds, wherein the said cover consists of spherical particles of diameter 0.05—0.5 mm, preferably 0.1—0.3 mm, based on derivatized cellulose with immobilized enzymes of the protease type selected from the group comprising chymotrypsine, trypsine and subtilisine.

2. Method for producing the proteolytic cover for wounds according to Claim 1, wherein a regenerated bead cellulose swollen in water and never dried before with particle size 0.07—0.7 mm, preferably 0.14—0.4 mm, is perfectly freed of toxic impurities by washing and/or distillation with steam, activated for binding of enzymes, modified by the immobilisation of proteases selected from the group comprising chymotrypsine, trypsine and subtilisine, alternately washed with a buffer of pH 8.5 to 9.5 and a buffer of pH 4 to 5 until the proteolytic activity of washing liquid is zero, then washed with a buffer of pH 7.5 to 8.5, and dried in this medium up to 0.1 to 15% of the residual water content in the dry substance, preferably by lyophilisation.

3. Proteolytic cover for wounds as claimed in Claim 1 substantially as described in any one of the examples disclosed herein.

4. Method of producing the proteolytic cover as claimed in Claim 2 substantially as described in any one of the examples disclosed herein.

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